EXHIBIT 6

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Methods

Total Chondroitin Sulfate Sodium by Cetylpyridinium Chloride Method
INA Method 120.002

Assay Title: Determination of chondrotin sulfate sodium polymer by cetylpyridinium chloride method.

Background: Chondroitin sulfate (CS) is the predominant glycosaminoglycan found in cartilage. Molecules are linear copolymers of variable lengths comprised of the repeating disaccharide, ß-glucuronic acid-(1–>3)-ß-N-acetylgalactosamine-(1–>4), in which the latter sugar is usually sulfated at either the 4 or 6 position. CS-4-sulfate and CS-6-sulfate are also known as chondroitin sulfates A and C, respectively, while the generic term chondroitin sulfate usually connotes a mixture of both isomers. It is important to understand that unlike other biopolymers that are unique molecular entities (e.g., proteins and nucleic acids), CS is not a single molecular species. The multiple reactions of biosynthesis (e.g., chain length extension, amination, N-acetylation, oxidation to uronic acid, location and extent of sulfation) ensue randomly and without the proofreading processes characteristic of template synthesis. An additional level of variability is introduced during extraction due to widely variable conditions of proprietary manufacturing processes (e.g., temperature and pH extremes, latent esterase activity in proteases, shear). CS therefore exhibits truly astronomical permutations of chemically similar species that lend themselves poorly to chromatographic resolution.

The analytical chemist is therefore challenged to distinguish this multitude of CS species from all other components of a potentially complex matrix. This selectivity can be simply and quite efficiently achieved by titration of CS with cetylpyridinium chloride (CPC) that forms water insoluble ion pairs principally (though not exclusively) with the sulfate moiety. The turbidimetric endpoint is determined by phototrode instrumentation and quantitation is by comparison of endpoint to that of CS reference standard. CS may originate from cartilage of bovine, porcine, avian, shark, or virtually any source, but the method cannot distinguish the animal or tissue origin. Monomeric and very small oligomeric CS species may not score by this method.

The purity of CS should not be concluded until a test intended to detect impurity is performed (electrophoresis or near infrared spectroscopy). Organic anions such as surfactants or some polymers (e.g., proteins and dextran sulfate) of sufficient molecular weight can interfere. Other CS congeners (usually representing a small fraction of the total) such as non-CS sulfated glycosaminoglycans (e.g., heparin, keratan sulfate and dermatan sulfate) and polycarboxylic nonsulfated glycosaminoglycans also score positively. Once raw materials are screened for identity, the method is remarkably insensitive to interference and can quantify CS in relatively complex matrices.

Safety: Consult the Material Safety Data Sheet (MSDS) for any chemical used that is unfamiliar. All chemicals should be considered hazardous - avoid direct physical

contact. For more safety information go to http://hazard.com/msds/.

Standards: CS is available from many suppliers, while availability of quality reference standard is limited. Sigma material (Catalog #C8529) is among the best characterized, has been used by laboratories for several years, and was utilized in this method. It is critical that only properly characterized chondroitin sulfate sodium reference standard be used.

NOTE: It has been established recently in multiple labs that Sigma's material assays at potency several percent greater than historically. Since the release of Sigma lot #71K2049 in August 2001 (and subsequent lots), this material assays by the INA method as being ultra-pure grade.

Apparatus:

- Autotitrator Colorimeter with probe (phototrode); preferentially with blue pass filter, generally 420 nm (sensitivity decreases slightly with increasing wavelength)
- Stirrer (if not integral with autotitrator)
- Calibrated analytical balance accurate to ±0.1 mg
- Flask, volumetric, Class A, assorted sizes
- pH meter, calibrated

Reagents:

- Water, HPLC grade or Nanopure
- Cetylpyridinium chloride, 98%, Aldrich, Cat. No. 22,899-0
- Sodium phosphate dibasic, ACS reagent grade
- Phosphoric acid, ACS reagent grade

Phosphate Buffer Preparation:

Dissolve 28.4 g dibasic sodium phosphate (Na_2HPO_4) in 800 mL water. Adjust to pH 7.2 (±0.1 pH) with 1 M phosphoric acid, then dilute to 1000 mL with water.

Cetylpyridinium Chloride Titrant Preparation:

Prepare a solution of cetylpyridinium chloride in water having a concentration of about 1 mg/mL.

Standard Preparation:

NOTICE: CS is extremely hygroscopic and MUST be dried prior to use. Dry a thin layer of standard in a crucible at 105°C for 4 hours. Transfer crucible to desiccator, cool, then quickly transfer to appropriate vessel with a tight seal. Store in desiccator at room temperature-**DO NOT REFRIGERATE**.

Accurately weigh (± 0.1 mg) 25 mg of CS reference standard into a 25-mL volumetric flask. [Note: CS mass will increase as it absorbs moisture while sitting

on the balance pan. Minimize atmospheric exposure and weigh quickly!] Dissolve in approximately 8 mL water, add 1.25 mL of pH 7.2 phosphate buffer solution, and dilute with water to volume. The resulting solution will have a known concentration of about 1 mg/mL.

NOTE: Although extremely soluble, CS is a gum. Some CS powders form surprisingly slow-dissolving particles or transparent films on vessel walls when mixed with water. Sonication is therefore recommended for both sample and standard preparations.

Sample Preparation:

Accurately weigh sample equivalent to approximately 100 mg chondroiton sulfate sodium and place in a 100-mL volumetric flask. [Similar precautions should be taken as described in Standard Preparation depending on whether samples are to be analyzed as is or dried.] Add 30 mL of water and stir until dissolved. Add 5 mL of pH 7.2 phosphate buffer solution, dilute with water to volume and mix. Analyze the sample on day of preparation.

Note: If analyzing a sample of low purity, increase sample size accordingly. If necessary, filter using 0.45 μm PTFE to improve signal. Linear dynamic range limit will be reached with samples of less than 10% purity.

Titration Parameters:

Parameters listed below are those employed by a Metrohm instrument that may be viewed as reasonable starting conditions. Other autotitrators are equally satisfactory.

1. Mode: Dynamic equivalence point titration, i.e., variable volume increments as function of the slope of the curve.
2. Titrant start volume (This is an initial amount to minimize analysis time, since typical COA analysis of CS raw materials range from 85 to 95%.)
    a. 4 mL (absolute)
    b. Dose rate: maximum
    c. 10 s post-start volume pause
3. 3. Post start volume
    a. Minimum volume increment: 75 μL
    b. Dose rate: maximum
4. Stop volume: 10 mL (absolute)
5. Evaluation
    a. End point criteria: 5 (only one real end point but baseline noise can be problematic.)
    b. End point recognition: Window (only end points that fall within window)
    c. Lower window limit: 150 mV
    d. Upper window limit: 700 mV

Procedure:

Transfer 5 mL of standard solution to the titration vessel and add about 30 mL of water. Titrate with cetylpyridinium chloride solution (cf. Titration Parameters, below) using a phototrode to determine the endpoint turbidimetrically, at 420 nm, with the instrument set in transmittance mode at 100% for the initial solution. Titrate the standard in triplicate.

NOTICE: The titration end-point does not ultimately exhibit a simple sigmoidal trace. As titration ensues, turbidity increases and transmittance decreases following a classical sigmoidal curve. It is the inflection of this curve that represents the end-point. As titration continues, however, excess CPC induces flocculation (i.e., the finely divided precipitate coalesces into larger particles) with appreciable recovery of transmittance. This post-titration aggregation is diagnostic that the end-point has been reached. While the end-point can be identified visually, the titrator's first-derivative option should be used (if available) to distinguish the aggregation phenomenon and more accurately identify the inflection.

Determine the equivalence factor, F, in mg of chondroiton sulfate reference standard per each mL of cetylpyridinium chloride solution by the formula:

$$F = 5(C/V_s)$$

Where:

C is the concentration (mg/mL) of chondroitin sulfate sodium reference standard

V_s is the mean volume (mL) of cetylpyridinium chloride solution consumed by the standard solution.

5 is the volume of standard solution used in the titration.

Transfer 5 mL of the sample preparation solution to the titration vessel and proceed with the titration as described above.

Calculations:

Calculate the percentage of total sulfated glycosaminoglycans as chondroitin sulfate sodium by the formula:

$$2000F(V_u/W) = \%w/w$$

Where:

F is the equivalence factor (mg/mL), calculated above.

V_u is the volume (mL) of cetylpyridinium chloride solution consumed by the sample preparation sample.

W is the weight (mg) of the sample

2000 is the dilution factor (100mL/5mL) x 100%

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Method Revision History:

120.001

Under "Titration Parameters" step 3, post start volume, the minimum increment was incorrectly listed as 75 mL. It was changed to 75 µL.

120.002

Note inserted describing improvement in Sigma's chondroitin sulfate purity.

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Standard Operating Procedure: #QA-061

USP CHONDROITIN SULFATE BY CPC TITRATION

1 Objective

1.1 To colorimetrically determine the content of chondroitin sulfate in raw material and finished product by precipitation with N-cetylpyridinium chloride (as applied to Metrohm autotitrator instrumentation).

2 Instrument/Apparatus

2.1 Autotitrator, Metrohm 751 GPD Titrino (or equivalent)
2.2 Stirrer, Metrohm 727 Ti Stand (or equivalent)
2.3 Colorimeter, Metrohm Model 910 w/ 420 nm interference filter and phototrode (or equivalent)
2.4 Assorted mixing and graduated glassware
2.5 Variable-volume micropipettes
2.6 Polypropylene culture tubes with caps, 14 ml
2.7 Magnetic stirrer and stir bars
2.8 Analytical balance
2.9 Stomacher with appropriate stomacher bags

3 Reagents

3.1 N-Cetylpyridinium chloride (CPC), 98%
3.2 Water, reagent grade, for analysis (≥1 MΩ, carbon- and submicron-filtered)
3.3 Water, deionized, for probe rinsing
3.4 Sodium phosphate, dibasic, reagent grade
3.5 USP chondroitin sulfate standard (or validated equivalent)
3.6 Phosphoric acid, reagent grade

4 Comments

4.1 CPC is a potential sensitizer, while both it and sodium phosphate are irritants. Phosphoric acid is corrosive. Inhalation and contact with eyes, skin and clothing should be avoided.
4.2 ALERT 1: If titrant line fittings are not sealed securely, air can enter during the buret's intake stroke. Diluted with bubbles, greater titrant volumes will return erroneously high results. Ensure all fittings are snug before operation.
4.3 ALERT 2: Chondroitin sulfate is exceedingly hygroscopic and is capable of absorbing several tens of percent of its own weight in

ambient moisture if exposed to the atmosphere, even on a day with relatively low humidity!

4.3.1 Mass determinations must therefore be performed as expeditiously as possible, and

4.3.2 All samples must be treated with the utmost care to ensure integrity and thorough sealing of all containers! If a retest is required of an improperly sealed sample, it will score low due to absorbed water.

5 Procedure

5.1 Assay mechanism (For assay procedure, cf. Section 5.5)

5.1.1 CS is an exceedingly water-soluble, high-molecular weight, polysulfated polysaccharide. CPC is a cationic detergent that ion-pairs with CS sulfates and neutralizes all charge. The solubility of oligomeric CS complexed with detergent is diminished but remains soluble. However, CS high polymers—enveloped in bound detergent—experience a local high density of hydrophobicity that promotes inter- and intramolecular aggregation with concomitant precipitation from solution.

5.1.2 Light scattering increases with precipitation and varies inversely proportional to transmittance. A monotonic drop in the latter is monitored by the photometer via phototrode until a break identifies the quantitative endpoint-point. With continued titrant addition the suspension turns flocculent with aggregation of microscopic to macroscopic particles as manifest by a partial increase in transmittance. The equivalence point is automatically calculated from the zero crossing of the second derivative of the titration curve.

5.2 Phosphate buffer [0.2 M sodium phosphate (pH 7.0)]

5.2.1 Transfer 28.4 g dibasic sodium phosphate to 1-l volumetric flask. Dissolve in ~800 ml water.

5.2.2 Adjust to pH 7.0 with dilute phosphoric acid.

5.2.3 QS to volume with water. [Storage for 30 days at 4° C.]

5.3 Chondroitin standard

5.3.1 Before use, dry 4 h in 105°C oven and allow to cool to room temperature in desiccator. Subsequently store desiccated at room temperature **(cf. hygroscopicity Alert #2, Section 4.3).**

5.3.2 Prepare standard at ca 1.00 mg CS/ml. Accurately weigh approximately 100 mg desiccated CS reference standard into a 100-ml volumetric flask. Dissolve in about 30 ml water, add 5 ml phosphate buffer (Section 5.2) and QS to volume with water; mix thoroughly.

5.4 Preparation of CPC titrant [0.1% CPC]

5.4.1 Accurately weigh approximately 1.0 g CPC and dissolve in 1 l water. Titrant may be used without further preparation and should be expired every 30 days.

5.4.2 Determination of CPC titrant factor

5.4.2.1 CS precipitation is a linear function of detergent concentration. However, lot to lot CPC composition and solution preparation is a source of potential assay variability. Each new CPC titrant preparation must therefore be calibrated against CS reference standard.

5.5 Sample analysis

5.5.1 Ready instrument

5.5.1.1 Turn power on at plug strip.

5.5.1.2 If not on, power-up colorimeter (toggle at upper right rear) and titrator (button at lower left of front panel). Both modules will perform a momentary self-test, and upon passing are ready for analysis.

5.5.1.3 With reservoir filled with titrant, purge lines as necessary by lowering probe into beaker and press DOS on front panel until syringe is filled and all air bubbles are purged.

5.5.1.4 Select method by pressing User Method (#3 key) select recall method, through ENTER. Select with the right/left arrows "ChondSTD;" press ENTER.

5.5.1.5 Set selector on colorimeter to percent transmittance (%T) and ensure 420 nm interference filter is secured in place.

5.5.1.6 Establish instrument control

5.5.1.6.1 If fresh titrant is prepared, calibrate first per Section 5.5.2.

5.5.1.6.2 If titrant is calibrated but analysis is the first run of the day, challenge the instrument with a control sample. cf. Section 5.5.2.3.

5.5.2 Calibration

5.5.2.1 Standards are analyzed at five levels CS concentrations: ranging from ca 4 to 5 mg (each accurately determined), corresponding to ca 80 to 100%, and plotted against the respective endpoint titrant volumes.

5.5.2.2 A line is regressed to the data with a correlation coefficient (r^2) of ≥0.995 (not forced through the origin), and the appropriate equation entered into the titrator for automatic calculation. [If $r^2 \leq 0.995$ occurs, a single point may be eliminated, but requires a tightened r^2 of ≥0.999 of the remaining four points!]

5.5.2.2.1 Equation entry

5.5.2.2.1.1 Press DEF (define, #2 key).

5.5.2.2.1.2 Select formula (highlight, then press enter).
5.5.2.2.1.3 Select equation #1 (press #1 key).
5.5.2.2.1.4 Using secondary key values, enter the equation: **RS1=(C01*EP1)+C02,** without spaces.
5.5.2.2.1.5 Press ENTER and it is seen that equation RS1 is assigned chondro, *4* decimal places, and the unit of *mg*, have been selected. Don't change these, but press QUIT to exit.

5.5.2.2.2 Enter constant values
5.5.2.2.2.1 Press C-FMLA (#1 key).
5.5.2.2.2.2 Assign C01 and C02 that are now options since present in the equation above.
5.5.2.2.2.2.1 C01 = m value of regression equation
5.5.2.2.2.2.2 C02 = b value of regression equation

5.5.2.3 Independent calibration verification
5.5.2.3.1 A new calibration may be linear, pass through the origin, and ostensibly correct when in reality it may be incorrect if the mass determination or initial dilution were incorrect. Therefore, an independent calibration verification (ICV) standard is prepared and with which the new calibration is challenged to verify its proper construction.
5.5.2.3.2 Freshly prep a second CS standard at a concentration that falls ca central to somewhat above central of the calibration, as described in Section 5.3.
5.5.2.3.3 Analyze this ICV against the new calibration.
5.5.2.3.4 The calibration is rejected if the ICV value differs by more than ±2% of that determined from the new calibration.

5.5.3 Sample preparation
5.5.3.1 Sample moisture
5.5.3.1.1 Samples received in ziplock bags should be analyzed promptly to minimize moisture absorption.
5.5.3.1.2 Sample handling should be expeditious to minimize absorption of atmospheric moisture.

5.5.3.1.3 LOD moistures are determined for every raw material receipt by either an infrared or halogen moisture balance method. These methods are validated against the oven LOD method and accounts for the material's proper mass balance.

5.5.3.1.4 Samples, therefore, can be analyzed *as is* and corrected to their *dry basis* value per the corresponding moisture determined by loss on drying.

5.5.3.2 Tablets, capsules, raw materials

5.5.3.2.1 Samples prepped should be representative, i.e., appropriately sampled and composited.

5.5.3.2.2 Accurately weigh sample equivalent to approximately 100 mg CS into a 100 ml volumetric flask.

5.5.3.2.3 Add ca 30 ml water and swirl to suspend. Then add 5 ml phosphate buffer and QS to volume with water.

5.5.3.2.4 Sonicate in 50° C bath for 20 minutes and cool in room temperature water bath.

5.5.3.2.4.1 **Samples thus prepared make ideal "fermentation media" and degrade quickly. Moreover, turbidity occasionally develops spontaneously upon standing for more than one hour. Samples must therefore be assayed no greater than 4 hour after they have been prepped or be expired.)**

5.5.3.2.4.2 **Samples generally need not be clarified (pelleted or filtered) since they are further diluted and the photometer is zeroed prior to analysis.**

5.5.3.3 Bars

5.5.3.3.1 Prepare a uniform composite of 12 bars (grinder or blender, do not soften in the microwave). Use 1/4 of each bar for the composite.

5.5.3.3.2 Weigh approximately 8 grams of sample into a stomacher bag.

5.5.3.3.3 Add 200 mL of water.

5.5.3.3.4 Stomach on normal for 60 seconds, then stomach on high for 120 seconds.

5.5.3.3.5 Briefly centrifuge prior to titration to minimize suspended matter.

5.5.4 Titration

5.5.4.1 Select the method: *Chondro*. Press *Card*, arrow to *Recall method*, ENTER. Arrow right or left to the select the method; press ENTER.

5.5.4.2 To a 50-ml beaker, add approximately 30 ml water, then accurately transfer an appropriate volume of sample with the glucosamine content targeted to the center of the calibration (ca 5 ml).

5.5.4.2.1 Add 250 µL of phosphate buffer to bar extracts, which are not buffered in sample preparation.

5.5.4.3 Lower phototrode/stirrer into beaker and set stirrer speed to rheostat setting 1.5 (red knob on stand). (NOTE: Phototrode/stirrer must be lowered fully to stop or stirrer will not turn.) If stirrer does not spin, press the stirrer button on front panel.

5.5.4.4 Allow colorimeter to equilibrate ~20 seconds, then press ZERO on the colorimeter panel, then press START.

5.5.4.5 *Sample identity*: The analyst is prompted for sample information. First select the alphanumeric option (the decimal key). With only an eight-character limit, enter as much of the sample name as is meaningful using the arrow keys and ENTER. Cancel the alphanumeric option with the QUIT key, then accept by pressing ENTER. Then enter the sample lot number using the keypad; accept with ENTER.

5.5.4.6 Titration will be performed, after which the titrant volume will be displayed, percent sample CS calculated, and both sample information and the titration curve will be printed. The first derivative curve will be superimposed on the titration curve, the peak of which identifies the endpoint-point. Tear printout from printer and staple to worksheet.

5.5.4.7 A single analysis is usually sufficient. If post-titration aggregation (cf. Section 5.1.2) does not occur, however, the endpoint (if determined) should not be trusted. The sample should be reprepped and assayed. If aggregation still does not occur, the sample should be prepped and rerun at a higher or lower concentration and reproducibility assured.

5.5.5 Subsequent sample runs and post-run cleanup

5.5.5.1 Raise and place beaker under phototrode/stirrer, then deliver several pumps of rinse water. Carefully wipe with tissue.

5.5.5.2 Empty titration beaker and rinse with water (squeeze bottle).

5.5.5.3 Set up next sample by repeating sections 5.5.4.2 to 5.5.4.7.

5.5.6 Check standards

5.5.6.1 Run a 90% CS check standard (calibration standard) following every six samples; terminate with same. **[Note: All check standards must be freshly prepped and used the same day!]**

5.5.6.2 Any check standard that differs by more than ±2% of the known check standard value, identifies the instrument as out-of-control and requires that all samples analyzed following the last passing check standard be failed and reanalyzed on a new verified calibration.

5.5.6.3 Instrument control must be reestablished, challenged with a control sample (and pass by above criteria!), and analysis of failed samples repeated.

6 Sample analysis program (Metrohm 751 GDP Titrino): "ChonSAMP"

Titration parameters

Meas. Pt. Density	4
Min. incr.	75 ul
Dos. Rate	max. ml/min
Signal drift	50 mV/min
Equilibr. Time	26 s
Start V:	abs.
Start V	4 ml
Dos. Rate	max. ml/min
Pause	10 s
Dos. Element	internal D0
Meas. Input	1
Temperature	25.0 C

Stop conditions

Stop V:	abs.
Stop V	10 ml
Stop U	OFF mV
Stop EP	1
Filling rate	max. ml/min

Statistics

Status	OFF

Evaluation

EPC	5
EP recognition	window
Low lim.1 U	700 mV
Up lim.1 U	150 Mv
Low lim.2 U	OFF mV
Fix EP1 at U	OFF mV
pK/HNP	OFF

Preselections

Req. ident	ID 1 & 2

Req.smpl size	Value
Activate pulse	OFF
Formula	
Chondro=(EP1*C01)+C02	
RS1 text	Chondro
RS1 decimal places	4
RS1 unit:	mg
Silo calculations	
Match id:	OFF
Common variables	--
Report	--
report COM1:full;comb;	
mean	--
temporary variables	--

8 Documentation

8.1 *VP-061: Validation Plan for the Analysis of Chondroitin Sulfate by CPC Titration* (QA-061)

8.2 Validation: Notebook MV-1, Minh Vu, pp. 49-72.